water

MDPI

Article

Effect of River Ecological Restoration on Biofilm Microbial Community Composition

Qiaoyan Lin[1], Raju Sekar[2], Rob Marrs[3], and Yixin Zhang[1,4,5,*]

[1] Department of Health and Environmental Sciences, Xi'an Jiaotong-Liverpool University, Suzhou, Jiangsu, China; qiaoyan.lin@xjtlu.edu.cn; yixin.zhang@xjtlu.edu.cn

[2] Department of Biological Sciences, Xi'an Jiaotong-Liverpool University, Suzhou, Jiangsu, China; sekar.raju@xjtlu.edu.cn

[3] School of Environmental Sciences, University of Liverpool, UK; calluna@liverpool.ac.uk

[4] Xi'an Jiaotong-Liverpool University Huai'an New Urbanization Institute, Huai'an, Jiangsu, China; yixin.zhang@xjtlu.edu.cn

[5] Xi'an Jiaotong-Liverpool University Suzhou Urban and Environment Research Institute, Suzhou, Jiangsu, China; yixin.zhang@xjtlu.edu.cn

***** Correspondence: yixin.zhang@xjtlu.edu.cn; Tel.:+86-512-88167109; Fax: +86-512-88161899

Received: date; Accepted: date; Published: date

Abstract: Across the world, there are increasing attempts to restore good ecological condition to degraded rivers through habitat restoration. Microbial communities developing as biofilms play an important role in river ecosystem functioning by driving organic matter decomposition and ecosystem respiration. However, little is known about the structure and function of microbial communities in riverine systems, and how these change when habitat restoration is implemented. Here, we compared the biofilm bacterial community composition using 16S rRNA genes targeted high-throughput Illumina Miseq sequencing in three river types, degraded urban rivers, urban rivers undergoing habitat restoration and forested rivers (our reference conditions). We aimed to determine: (i) the biofilm bacterial community composition affected by habitat restoration (ii) the difference in bacterial diversity in restored rivers, and (iii) correlations between environmental variables and bacterial community composition. The results showed that both water quality and biofilm bacterial community structure were changed by habitat restoration. In rivers where habitat has been restored, there has been an increase in dissolved oxygen, a reduction in organic pollutants, a reduction in bacterial diversity and a related developing pattern of microbial communities, which is moving towards that of the reference conditions (forested rivers). River habitat management stimulated the processing of organic pollutants through the variation in microbial community composition, however, a big difference in bacterial structure still existed between the restored rivers and the reference forest rivers. Thus, habitat restoration is an efficient way of modifying the biofilm microbial community composition for sustainable freshwater management. It will, however, take a much longer time for degraded rivers to attain the similar ecosystem quality as the "pristine" forest sites than the seven years of restoration studied here.

Keywords: bacterial community; biofilm; Illumina Miseq sequencing; habitat restoration; river ecosystem

1. Introduction

One of the current aims in riverine ecology is to use ecological restoration techniques to improve the quality of river ecosystem health, especially in urban areas where rivers have often been degraded severely [1]. Degraded rivers are normally formed by water pollution, land reclamation, dredging, channelisation, altered hydrology and the clearing of riparian zones [2, 3]. Ecological restoration approach aims to recover river habitat quality by increasing river habitat complexity and heterogeneity; this is achieved by reconfiguring the river channel, increasing flood plain areas, adding in-stream islands, and aquatic vegetation [1]; all designed to enhance the hydraulic and substrate heterogeneity and macrophyte colonization. In combination, these treatments should increase food availability within the ecosystem [4, 5], and eventually, a complexity of aquatic habitats (e.g. riffle, run, pool, and debris dam classifications) will develop in these restored rivers [6].

Healthy river habitats not only allow the living micro-organisms, aquatic flora (e.g. algae, aquatic plants) and fauna (e.g. macro-invertebrates, fishes) to persist, but they can also provide important ecosystems services, for example by reducing pollutants, such as organic matter, nutrients and heavy metals [7]. Riverine habitats are known to influence the diversity and composition of aquatic biotas through river morphology, hydrology, sedimentation, and by changing environmental variables at the reach scale, the latter important for larger stream organisms such as fish and macro-invertebrates [8]. For example, the surface features of the stream may influence detritus accumulation [9], and hence form 'refuges' for predators [10, 11]. Moreover, the habitat complexity generated by surface irregularities exerts a significant impact on the abundance and diversity of benthic invertebrates in stream systems [6, 12, 13]. In a meta-analysis, in-stream habitat heterogeneity restoration (including wood, boulder additions and channel reconfigurations) enhanced macro-invertebrate richness [6]. Nuttle et al., (2017) also found that cutting gates, restoring substrates, and enhancing in-stream and riparian habitats, significantly enhanced (i) the taxon richness of macro-invertebrates, and (ii) the richness and abundance of fish in 18 mitigation sites [15]. In spite of this, very little is known about the effects of river habitat restoration on the composition of biofilm microbial communities.

Biofilms, are a complex assemblage of microbial communities composed of bacteria, archaea, fungi, algae, and exopolysaccharides produced by the microorganisms. They are important components of stream ecosystems, and are considered a good bio-indicator of environmental health [16], not only because of their high abundance in most natural environments, but also because of their sensitivity to environmental changes with short life-cycle. Biofilms are a basic component of freshwater food webs; they adhere to the surfaces of rock particles and aquatic plants, and are influenced by many environmental factors including temperature, light, shear forces, nutrients and contaminants [17-19]. They fix energy and carbon by photosynthesis and chemosynthesis and some can also fix nitrogen [20]. They also recycle organic nitrogen, impact on dissolved organic matter, and play key roles in nutrient cycling, organic compound degradation, water quality remediation and suspended sediment removal [21]. Effectively, altering any environmental factor can affect stream biofilm communities, and this may in turn alter their function of the whole stream ecosystem [22]. Bacteria are an indispensable part of the epilithic biofilm, usually occupying 1-5% of the epilithic biofilm, and playing key roles in nutrient cycling, metabolic processes and many other biogeochemical processes and ecosystem functions [23-25]. The rates of bacterial-mediated nitrification, denitrification, and heterotrophic nitrogen (N) uptake in small streams have been shown to affect downstream water quality [25-27]. However, the impact of habitat restoration on biofilm bacterial community composition is still unclear.

To address this lack of information about biofilms during riverine restoration, we compared microbial populations in three different river types along a disturbance gradient. The most disturbed sites in this study were in urban areas, and the least disturbed sites were in forested catchments. In between, were rivers in urban areas where the habitat had been restored within the last seven years

as part of an ecological restoration strategy. We measured a range of environmental factors and assessed the microbial community using a standardized field procedure followed by 16S rRNA Illumina MiSeq. Through comparing the relationship among habitat status, environmental parameters and bacterial community composition, we aimed to determine: (i) the biofilm bacterial community composition affected by habitat restoration (ii) the difference in bacterial diversity in restored rivers and urban degraded rivers, and (iii) any correlations between bacterial community composition and selected environmental variables. We hypothesized that habitat restoration would alter the biofilm bacterial community composition in these restored rivers compared to the degraded ones and that they would become similar to the reference forest rivers. The bacterial diversity would be shifted toward near-natural state where habitat had been restored. The substrate composition and physico-chemical variables like dissolved oxygen, nutrient and organic pollutant might be leading factors affecting the bacterial community composition in river groups.

2. Materials and Methods

2.1. Study Sites

This study compared three stream types in the winter of 2017: (i) degraded rivers in urban areas, (ii) restored rivers, where an aquatic habitat restoration scheme had been implemented within the last seven years for each river; and (iii) rivers in forested catchments as reference conditions. Nine streams with similar-sized watersheds within the Anji City Region, Zhejiang Province PRC were selected for this study (Figure 1, Table S1). There were three replicates of each stream type, all located in different places in Anji City. The average day/night temperatures of the region were 12 ℃/5 ℃ in winter, and an average precipitation of 50 mm.

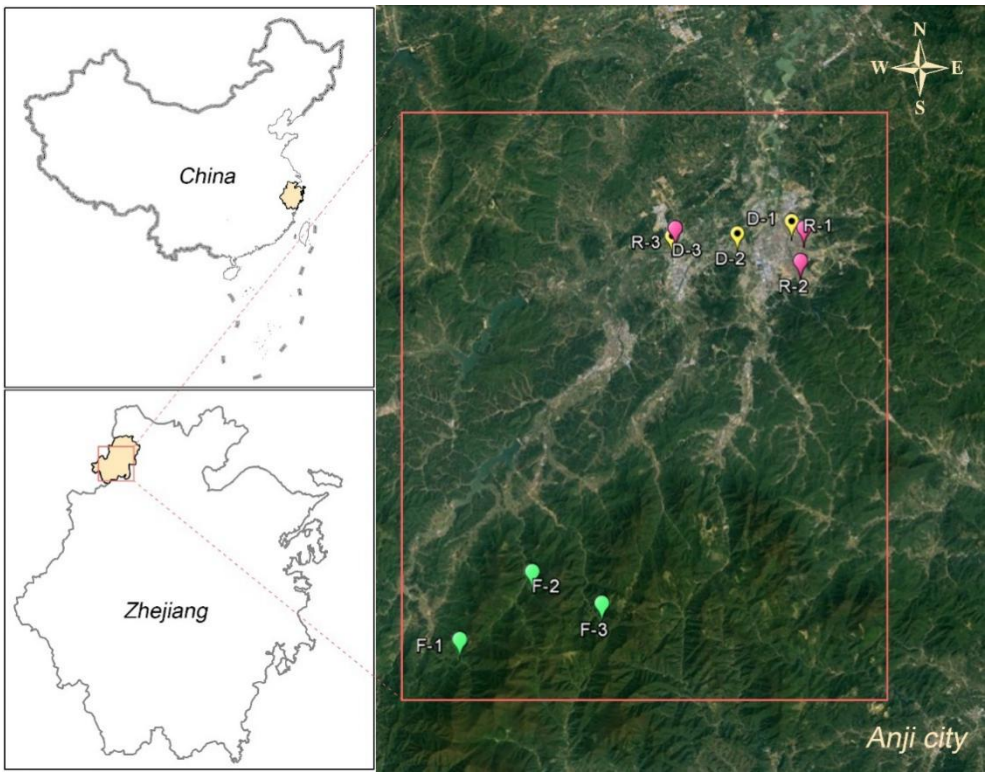


Figure 1. Location of the sampling sites within the Anji City Region, PRC; Containing three degraded urban rivers (D), three restored rivers (R) and three Forested rivers (F). The three forest streams (F) were upstream from Anji City; the three restored rivers (R) and the three degraded urban rivers (D) were downstream of the forest ones.

The three urban degraded sites (denoted D) were similar to the pre-restoration status of our restored rivers, Tongxin River is located in the city center, and the other two are located in the suburban districts. The three restored rivers (denoted R) have been restored for up to seven years using a mixture of ecological restoration techniques to reconstruct a natural river form. The techniques used included channel re-meandering, creation of riffles, pools and run areas, construction of floating islands, aquatic plant re-introduction, and riparian zone afforestation. A subsidiary aim was to provide ecosystems that could be used for ecological research, education and entertainment. Three forest streams (denoted F) were in the Tianmu Mountains (maximum elevation 590 m), 40-km upstream from Anji City were set as our "reference" conditions, because pristine rivers were not available in the city area. There has been relatively little human interference on these forest streams, and they represent pre-urban landscape form where the urban rivers have derived [28].

2.2. Habitat Survey and Physico-chemical Parameters of Stream Water

Habitat surveys were performed in December 2017 and January 2018. Reach canopy cover was estimated visually and presence of various mesohabitat counted (island, pool, riffle). To estimate the variation of sediment grain size within each reach studied, 100 sediment particles were selected randomly on the river bed and proportions of boulders (> 256 mm in diameter), cobbles (64-256 mm), pebbles (4-64 mm) and sand grains (2-4 mm) were counted [29]. The substrate diversity was calculated using the percentage cover of all substrate classes using the Shannon diversity index H' [30] for each study site.

Thereafter, within each river, the river width was measured using a 100 m tape. Water velocity and river depth were measured at five evenly-spaced points across the channel using Teledyne flow meters (ISCO, Lincoln, Nebraska, USA) and a steel ruler. Water quality in each river was monitored at three different points with 3 m interval at the maximum by *in situ* measurement of temperature, pH, both using a HACH pH/temperature meter (HACH, LA-pH 10, USA), dissolved oxygen (DO), using a YSI Professional Plus probe (YSI Propolus, USA), and turbidity, using a turbidity meter (HACH, DR2100Q, USA). A 1 litre water sample was collected from each stream and filtered in the field through 0.45 um Jingteng syringe tip filters and preserved at 4 ℃ before sending to the laboratory. These water samples were analyzed within 48 hours for (i) total nitrogen (TN) and total organic carbon (TOC), measured using a total organic carbon analyzer with a total nitrogen module (Multi N/C3100, analytik-jena, German), (ii) ammonium nitrogen (NH_4^+), nitrate-nitrogen (NO_3^-), and total phosphorus (TP), measured using a QuickChem® Flow Injection Analysis system (Lachat Instrument, Hach, USA), and (iii) chemical oxygen demand (COD), measured using a DR1010 COD analyzer (HACH, USA).

2.3. Biofilm Sampling Procedure

Biofilm was sampled by placing four 10 cm × 10 cm autoclaved unglazed tiles, at 0.3 m water depth in each river for 39 days; thereafter the biofilms were collected by scraping the accumulated materials from the tiles into 50 ml tubes covered with aluminum foil, and transported in a cool box to the laboratory. The material in each 50 ml tube was then separated into two, one part was filtered through 0.45 um membrane filter (Jingteng) to measure chlorophyll *a* (Chl-*a*) using a fluorimeter (10AU, Turner Designs, Sunnyvale, California, USA) after acetone extraction [31], and the other part was filtered on 0.22 μm pore size polycarbonate membrane filters (Millipore, USA) using a vacuum pump; these filters were stored in sterile Petri dishes at -20 °C until DNA extraction.

2.4. DNA Extraction and Analysis of Bacterial Community Composition

The genomic DNA of all the biofilm samples was extracted using DNA extraction Kit (MO BIO PowerBiofilm® DNA Isolation Kit, USA) based on a standard protocol. The DNA concentration was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the

ratio of absorbance at 260 nm and 280 nm checked to insure the quality of DNA obtained. All DNA samples were then preserved at -80 ℃ before processing for bacterial community analysis.

The bacterial diversity and community composition of all biofilm samples were measured using the Illumina Miseq sequencing at Suzhou Genewiz Company. Using 30-50 ng DNA as the template, the 16S rRNA genes covering the V3-V4 regions were first amplified from the DNA extracts using the forward primer 347F "CCTACGGRRBGCASCAGKVRVGAAT", and the reverse primer 802R "GGACTACNVGGGTWTCTAATCC". PCR amplification was conducted in triplicate for each sample using 25 μl PCR reactions mixture containing 2.5 μl TransStart Buffer, 2 μl dNTPs, 2 μl of each primer, 0.2 μl BSA, 0.4 μl FastPfu DNA polymerase, 20 ng DNA template and ddH_2O. PCR was performed using the following conditions: initial denaturation at 95 ℃ for 3 min, 24 cycles of denaturation at 94 ℃ for 30 s, annealing at 57 ℃ for 90 s, and extension at 72 ℃ for 10 s. The PCR amplicons were checked by 2% agarose gel electrophoresis and purified using MagPure Gel Pure DNA Mini Kit (Magen). The purified amplicons were pooled and paired-end sequenced on the Illumina MiSeq platform (Illumina, USA) at a read length of 2 × 300 bp.

After 16S rRNA sequencing, the reads were sorted to the samples according to barcodes, and the barcodes and primers were then removed. The low-quality reads were discarded, including the reads which did not exactly match the primer, the reads containing ambiguous character (N), a sequence length <200 bp, and reads with an average quality score <20. Then chimeric sequences were detected and removed by comparing the sequences with the reference database (RDP Gold database) [32] using UCHIME algorithm [33]. The high-quality sequences were clustered into operational taxonomic units (OTUs) using the clustering program VSEARCH9 (1.9.6) against the Silva 128 16S rRNA database with 97% sequence identity threshold. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic category to all OTUs at a confidence threshold of 0.8. The 16S rRNA gene sequences were submitted to National Centre for Biotechnological Information (NCBI) Sequence Read Archive database under the accession numbers MH889163 - MH890450.

2.5. Statistical Analysis

We evaluated differences in habitat characteristics, physico-chemical features, bacterial diversity and richness in different stream types (forest, urban restored and degraded) using one-way analysis of variance [34], followed by the Tukey's HSD post-hoc test for comparison of means. Pearson correlation coefficients were used to explore relationships between environmental parameters and all microbial variables. Differences were accepted as significant at $p = 0.05$ level. These statistical analyses were performed in the R statistical Environment [35].

Based on the results of the operational taxonomic units (OTUs) analysis, α-diversity indices (Shannon-Weiner index; Chao1 richness) were calculated in QIIME1.9.1 [36]. Non-metric Multi-dimensional Scaling (NMDS) plot was performed to display β-diversity based on Euclidean dissimilarities between each samples using the 'vegan' package [37] within the R statistical Environment [35]. Analysis of similarities (ANOSIM) was then performed to evaluate the bacterial community similarity among three river types using the vegan package. Venn diagrams were drawn to analyze overlapped and unique OTUs of each sample based on cluster analysis of OTUs. Metastats [38] was performed to detect the differentially abundant taxonomic groups at phylum and genus levels between different river types. The relationships between the bacterial community and environmental parameters (pH, turbidity, DO, TN, TP, TOC, NH_4^+-N, NO_3-N and COD) were assessed using redundancy analysis (RDA) within Canoco 4.5 for windows [39].

3. Results

3.1. Habitat Characteristics

There was a significant difference in canopy cover among the different river types ($F_{2,6}$ = 13.435, p = 0.006); canopy cover was significantly greater in forest rivers, intermediate in degraded rivers, and lowest in restored rivers. Forest and restored rivers had greater diversity of river bed habitat types than degraded rivers. In forest and restored rivers, riffles, pools, islands were commonly found whereas in the degraded rivers only pools, and a few islands were observed. In terms of substrate composition, the Shannon diversity (H') of substrate (ranging from 0 to 1.13) was significantly greater in forest and restored rivers (p = 0.001) and lowest in degraded rivers. Only granules were found in degraded rivers, whereas the restored and forest rivers had boulders (forest-only), cobbles, pebbles and granules. Degraded sites had much smaller substrates, whereas restored and forest rivers had bigger substrates.

3.2. Effects of Habitat Restoration on Physico-chemical Properties of Stream Water

Physico-chemical values (Table 1a) revealed no significant differences among river types for river width ($F_{2,6}$ = 0.336) and mean depth ($F_{2,6}$ = 0.791), and no difference in the surface water for pH ($F_{2,6}$ = 1.815), NH_4^+ ($F_{2,6}$ = 1.533), NO_3^- ($F_{2,6}$ = 0.374), TN ($F_{2,6}$ = 2.708), TP ($F_{2,6}$ = 0.042) and COD ($F_{2,6}$ = 5.069). However, significant differences were observed in surface water properties among the stream types for DO ($F_{2,6}$ = 7.398, p = 0.024), turbidity ($F_{2,6}$ = 7.69, p = 0.022), TOC ($F_{2,6}$ = 17.86, p = 0.003) and Chl-*a* ($F_{2,6}$ = 8.94, p = 0.016). The forest and restored rivers had similar concentrations of DO, and both had significantly greater DO concentrations than the degraded rivers (p < 0.05) (Figure 2A). The turbidity in degraded rivers was much greater than forest rivers (p = 0.018), while no differences were observed between forest rivers and restored rivers, restored rivers and degraded rivers (p > 0.1) (Figure 2B). Degraded rivers and restored rivers had greater TOC concentrations than forest rivers (p = 0.002 and p = 0.027, respectively). Although no significant difference was detected when comparing restored rivers with degraded rivers (p > 0.1), a reduction in TOC concentration was observed (Figure 2C). In terms of Chl-*a*, no differences were detected when comparing forest rivers with restored rivers and degraded rivers (p > 0.1), whereas rivers under restoration had a much higher Chl-*a* concentration than degraded rivers (p = 0.013) (Figure 2D).

Table 1. Mean values of (a) physico-chemical variables and (b) microbial diversity in different types of rivers within the Anji City Region, PRC. The values represent the mean ±standard error of three replicate samples.

(a)

River Type	**Width (m)**	**Mean Depth (cm)**	Dissolved Oxygen (mg/l)	pH	Turbidity	NH4-N (mg/l)	NO3-N (mg/l)	Total N (mg/l)	Total P (mg/l)	Chemical Oxygen Demand (mg/l)	Total Organic C (mg/l)	Chlorophyll *a* (mg/l)
Forest	8.83± 1.64	35.87± 7.97	14.16± 0.80	7.33± 0.11	0.62±0.14	0.02± 0.01	1.06± 0.13	1.99± 0.21	0.18± 0.02	2.44±0.15	0.48± 0.16	0.61±0.23
Restored	13.17± 3.09	28.13± 7.22	13.14± 0.65	7.64± 0.14	3.52±0.85	0.08± 0.02	1.13± 0.40	2.74± 0.77	0.17± 0.02	3.35±0.76	2.81± 0.32	1.22±0.19

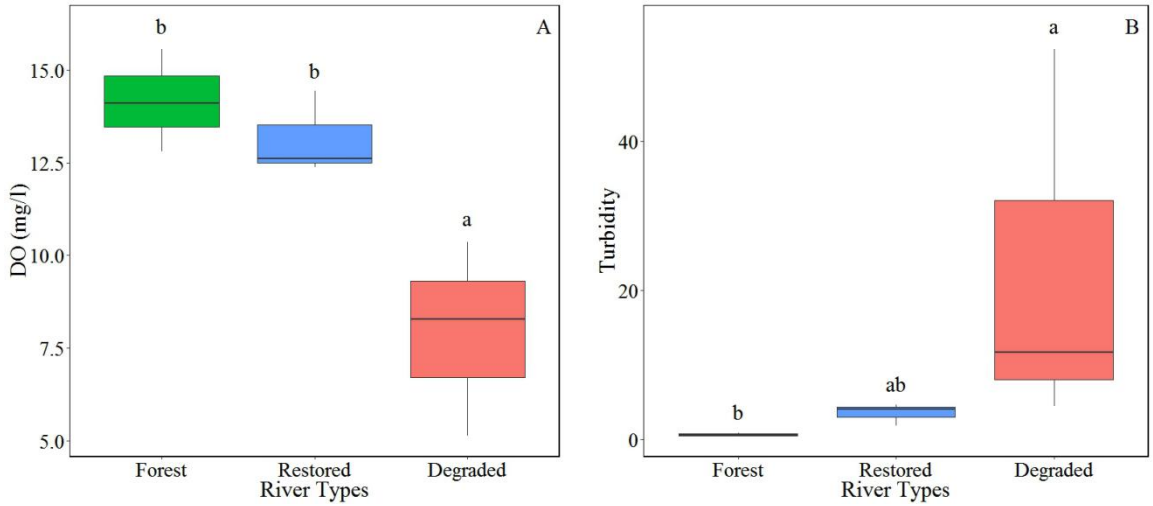
Degra	11.57±	22.87±	7.91±1.	7.38±	22.81±1	1.37±	0.79±	4.01±	0.18±	8.82±3	6.70±	0.20±0.0
ded	5.72	3.86	52	0.11	4.93	1.19	0.40	0.76	0.05	.40	2.21	9

(b)

River Type	Observed OTUs	Unique OTUs	Diversity Indices	
			Chao 1 Value	Shannon-Weiner Index
Forest	604.11 ±38.87	14.67 ±0.88	715.45 ±36.27	6.42 ±0.12
Restored	585.00 ±19.86	5.67 ±3.18	708.84 ±21.18	5.89 ±0.15
Degraded	666.89 ±69.17	30.00 ±14.80	769.73 ±72.81	6.98 ±0.17

3.3. Effects of Habitat Restoration on Bacterial Community Composition

A total of 3,300,566 reads were obtained from the 27 samples. After filtering, denoising, and chimera removal, 1650283 high-quality 16S rRNA gene-reads were obtained, ranging from 48,473 to 69,662 reads per sample. Mean OTUs and α-diversity values (Table 1b) showed that bacterial diversity measured by Shannon diversity index (H') was different between the river types ($F_{2,6}$ = 14.067, p = 0.005), being significantly greater in degraded rivers ($F_{2,6}$ = 6.98, p = 0.004) than restored rivers, whereas no distinct difference was found between restored rivers and forest rivers with respect to bacterial diversity (Figure 2F). Bacterial richness (Chao 1 Index) varied from 629 to 874, however, no significant differences were detected among river types for bacterial richness (Figure 2E).



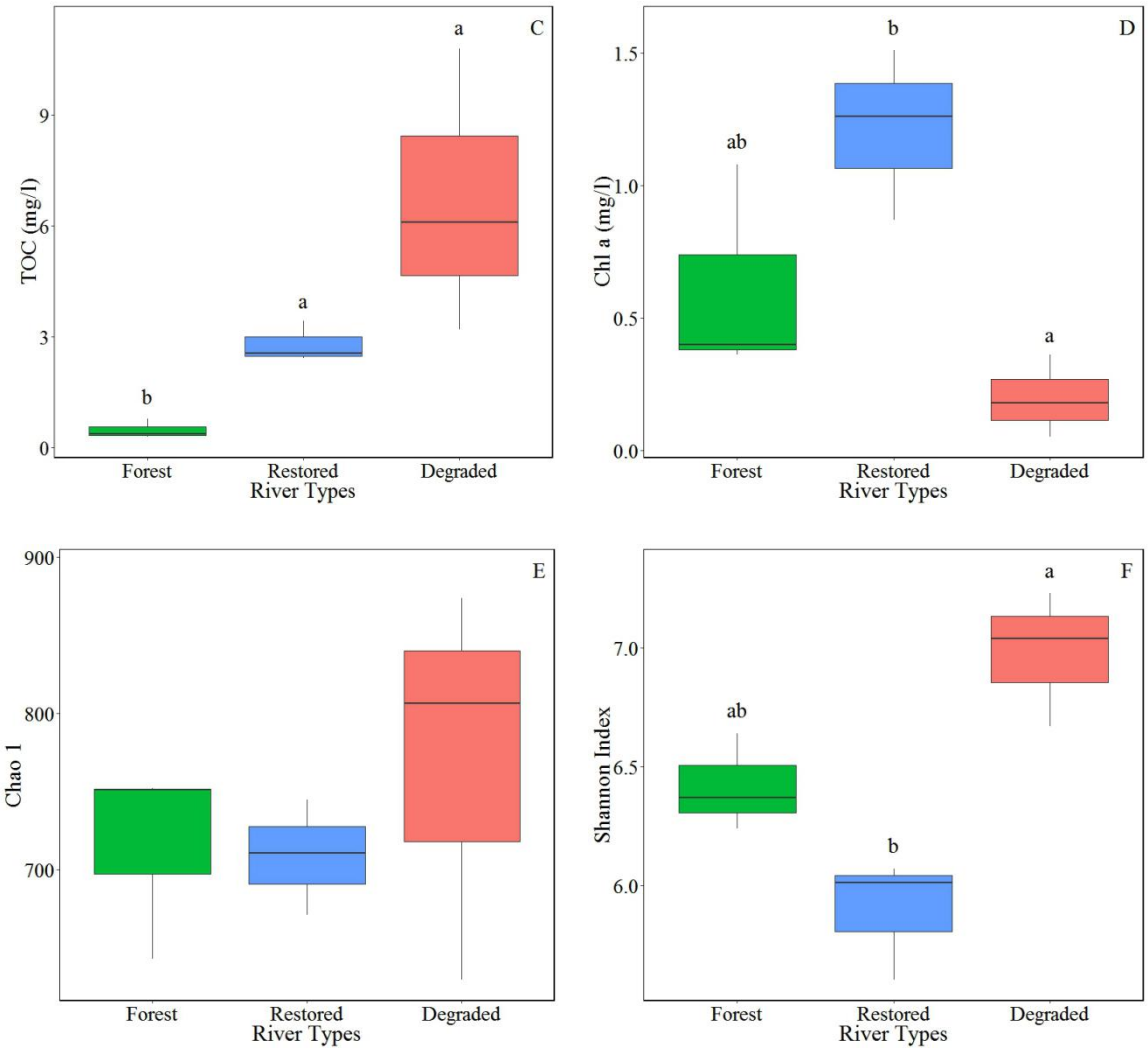


Figure 2. Boxplots representing the variance of physico-chemical parameters (A) dissolved oxygen (DO), (B) turbidity, (C) total organic carbon (TOC), (D) Chl-*a* and bacterial *α*-diversity (E) bacterial richness (Chao 1 Index), (F) bacterial diversity (Shannon Index) in forested, restored and degraded rivers within the Anji City Region, PRC. Black line: median value; box: quartile interval; whiskers: minimum and maximum value. Different lowercase letters indicate the significant difference observed at p = 0.05 level.

The NMDS analysis produced a stress value <0.094, indicating that the ordination produced a good summary of the observed distances between samples with obvious clustering (Figure 3). The bacterial community structures among all three river types were distinct from each other (R = 0.508, p = 0.001) as shown by analysis of similarities (ANOSIM) (Table 2). Although there was some overlap between restored and degraded rivers, the bacterial community composition was significantly different (R = 0.256, p = 0.008) and there was a clear shift in bacterial community composition along the first axes from degraded to restored rivers, and from restored to forest rivers.

Table 2. Analysis of similarities (ANOSIM) of biofilm bacterial communities in contrasting river types within the Anji City Region, PRC.

River-type Comparison	ANOSIM	
	R	*p*

Forest vs. Degraded	0.645	0.001
Forest vs. Restored	0.733	0.001
Restored vs. Degraded	0.256	0.008

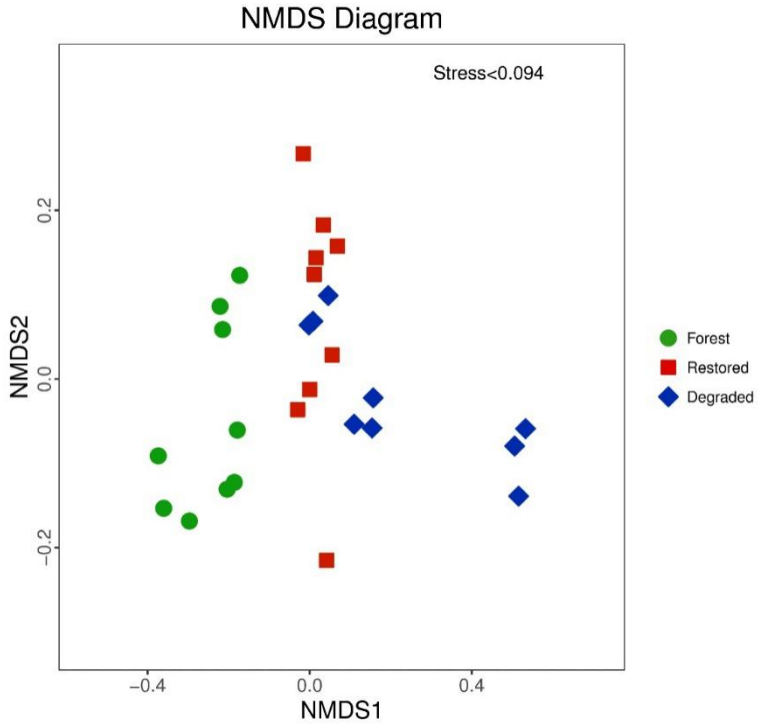


Figure 3. Non-metric Multi-dimensional Scaling (NMDS, stress<0.094) ordination of biofilm bacterial communities in forested, restored and degraded rivers within the Anji City Region, PRC within the Anji City Region, PRC.

In total, 383 OTUs were detected, 232 OTUs (61%) of which were universally present from biofilms in all rivers, and the three types of rivers contained 11.5% (forested), 4% (restored) and 23% (degraded) unique OTUs, respectively (Figure 4). The degraded rivers had greater percentage of unique OTUs, including genera Rhodocyclales, Cytophagales, Sphingobacteriales, however, no statistical differences were detected among river types for unique OTUs ($F_{2,6} = 2.81$).

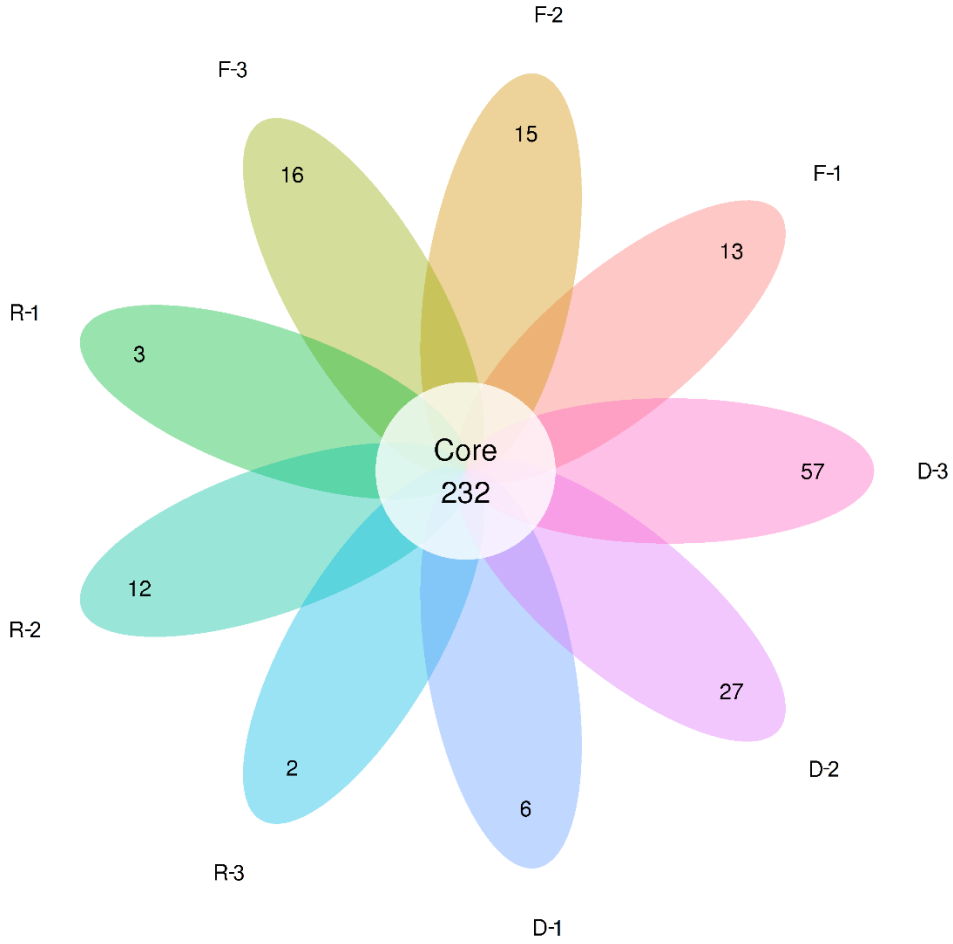


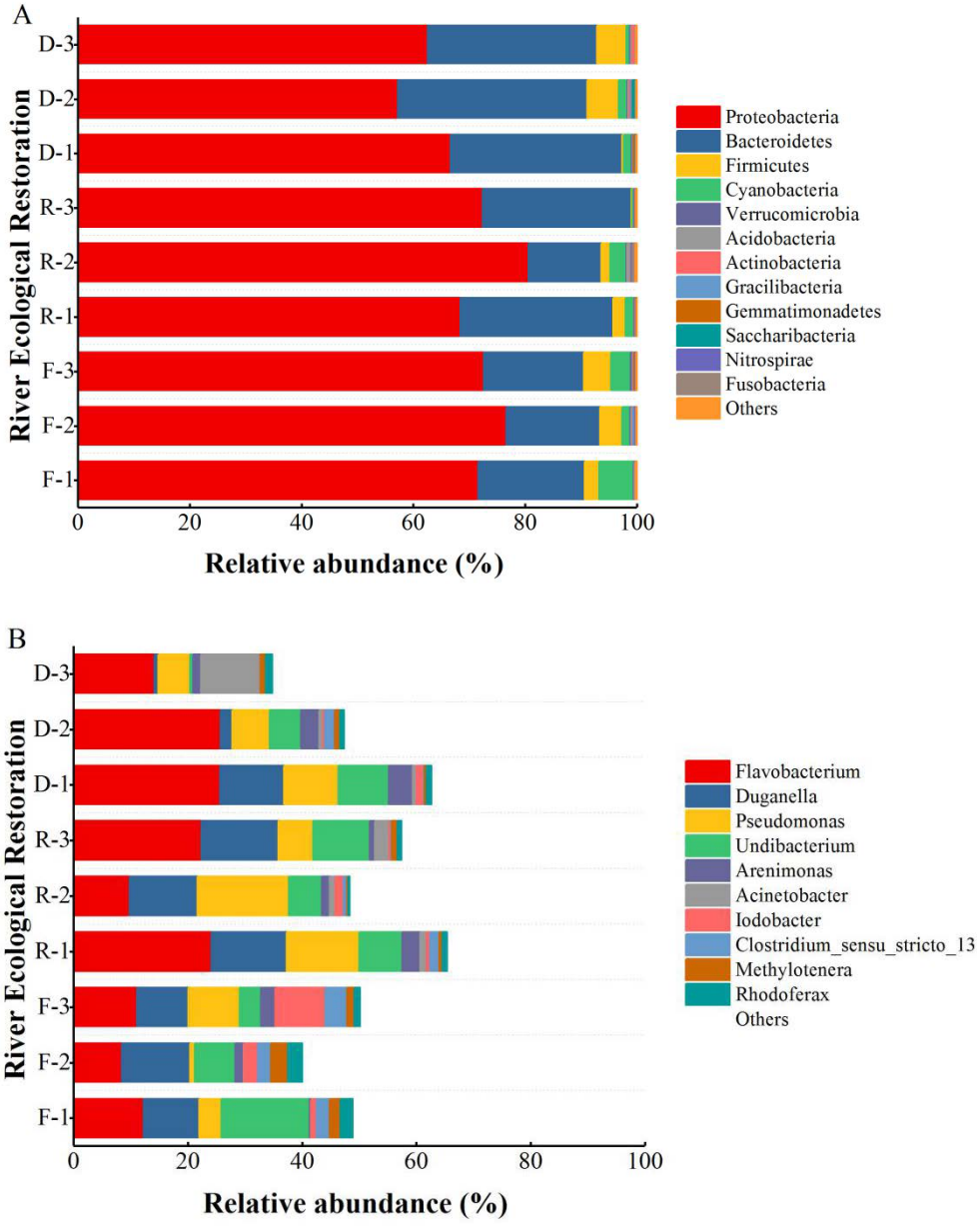
Figure 4. Venn diagram showing the number of unique and shared Operational Taxonomic Units (OTUs) among biofilms in forested (F), restored (R) and degraded (D) rivers within the Anji City Region, PRC.

The relative abundance of the bacterial community was calculated respectively both at phylum and genus level. At phylum level (Figure 5A), Proteobacteria was the most abundant phylum in all rivers, followed by Bacteroidetes, Firmicutes, Cyanobacteria, Verrucomicrobia, Acidobacteria and Actinobacteria. Rivers in forest and after restoration had a greater Proteobacteria abundance than degraded rivers ($p = 0.050$, $p = 0.049$, respectively), while no difference was detected between forest and restored rivers ($P > 0.05$). The relative abundance of bacteria in the phylum Bacteroidetes, a genera commonly assumed to be specialized in degrading high molecular weight (HMW) compounds [40], was slightly greater in degraded rivers than forest rivers ($p = 0.064$), while, no differences of Bacteroidetes were observed when comparing forest rivers with restored rivers, and restored rivers with degraded rivers ($p > 0.01$).

In terms of relative abundance at genus level, *Flavobacterium*, *Duganella*, *Pseudomonas*, *Undibacterium* and *Arenimonas* were commonly distributed in all studied rivers (Figure 5B). Degraded rivers showed significant numbers of reads allocated to *Flavobacterium* ($p = 0.001$), *Arenimonas* ($p = 0.026$) and *Acinetobacter* ($p = 0.001$). Forest rivers had a higher relative abundance of *Duganella* ($p = 0.022$), *Indobacter* ($p = 0.010$), *Clostridium_sensu_stricto_13* ($p = 0.006$), *Methylotenera* ($p = 0.001$) and *Rhodoferax* ($p = 0.007$) than degraded rivers. Among restored rivers, a greater relative abundance of *Flavobacterium*, *Pseudomonas*, *Acinetobacter* and a lower relative abundance of *Indobacter*, *Clostridium_sensu_stricto_13*, *Methylotenera* and *Rhodoferax* ($p < 0.05$) was found when comparing restored rivers with forest rivers. Restored rivers had a greater relative abundance of *Duganella* ($p =$

298 0.023) than degraded rivers. No difference in genus abundance was found between restored and
299 degraded rivers for other taxa.

300



301

302

303 **Figure 5.** Relative abundance of bacterial community at Phylum (A) and Genus level (B) in forested
304 (F), restored (R) and degraded (D) rivers within the Anji City Region.

305 *3.4. Correlation between Bacterial Community Composition and Environmental Variables*

306 Bacterial richness (OTUs) showed a positive correlation with water turbidity and a negative
307 correlation with TP concentration ($p = 0.049$, $p = 0.032$, respectively). Bacterial diversity showed a
308 strong positive correlation with water turbidity ($p = 0.006$), COD ($p = 0.023$), and TOC concentration
309 ($p = 0.019$), and was negatively correlated with substrate diversity ($p = 0.033$). The relationship
310 between environmental parameters and the total bacterial community composition was further
311 evaluated by constrained redundancy analysis (RDA), which produced eigenvalues for the first two
312 axes of 0.322 and 0.159, respectively (Figure 6). The environmental variables explained 48.1% of
313 bacterial community structure variance. The biofilm bacterial assemblages in forest rivers were

positively correlated with substrate diversity (r = 0.156) , and Chl-*a* concentrations (r = 0.828), and were negatively affected by NH_4^+ (r = - 0.621) and COD (r = - 0.629) of surface water. The reverse pattern was found for biofilms in the degraded rivers, COD (r = 0.999), TOC (r = 0.984), NH_4^+ (r = 0.738) and TN (r = 0.635) in the surface water presented as major factors linking to the bacterial structure in degraded rivers. For the restored rivers, the bacterial samples showed positive correlations with DO (r = 0.571) and substrate diversity (r = 0.652), and was affected negatively by COD (r = - 0.522) and NH_4^+ (r = - 0.526), though the correlations were not as strong as the forest rivers.

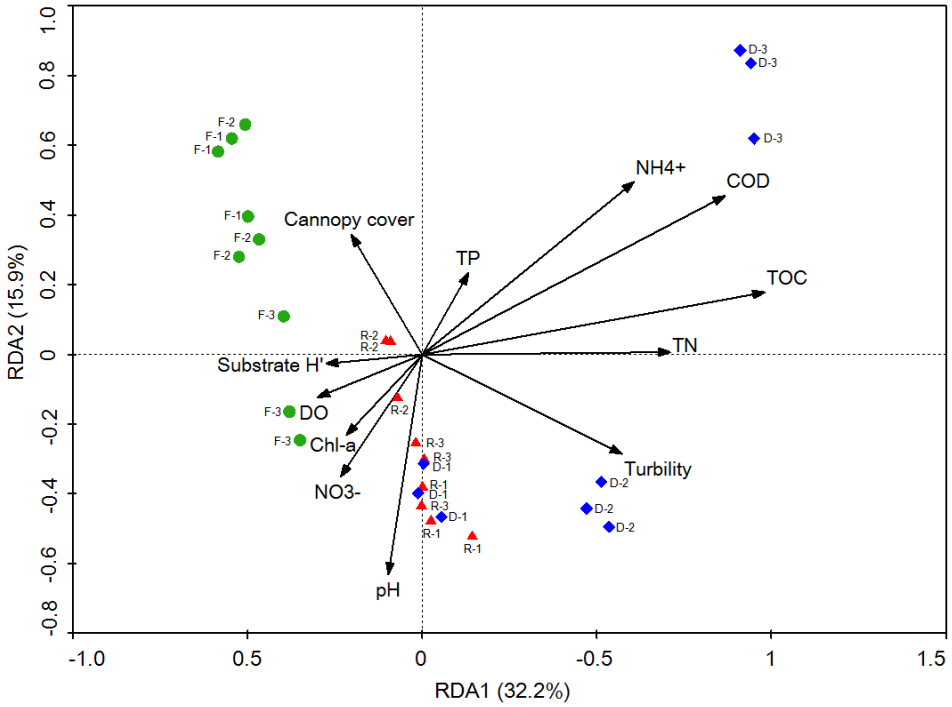


Figure 6. Relationship between the biofilm bacterial community and environmental variables in forested (F, circles), restored (R, triangles) and degraded (D, diamonds) rivers within the Anji City Region, PRC.

4. Discussion

Rehabilitation of aquatic biota, through habitat restoration, is now being implemented around the world to prevent further damage and mitigate existing freshwater degradation [41]. Accumulating evidence has linked aquatic rehabilitation to reducing nitrogen, phosphorus and organic matter concentrations, and thereafter to improved conditions for macro-invertebrate and fish populations [6, 15, 42]. Microbial communities are often ignored in stream restoration studies yet they are crucial for supporting aquatic ecosystem processes and functions with key roles in driving organic matter and nutrient cycling [43]. It is, therefore, imperative that we obtain a better understanding of the underlying mechanisms of microbe-mediated processes. In this study, therefore, we described the bacterial community composition including those involved in important ecological functions in restored rivers, and compared them with both degraded urban sites and "pristine" reference forest sites; to do this we used high-throughput 16S rRNA gene amplicon sequencing methods. The results showed clear differences in the structure of biofilm microbial communities among these three main river ecosystems, and these differences were strongly correlated to the changes in habitat and physico-chemical characteristics in these river groups. This finding is consistent with the results of surveys in New Zealand and USA, showing that local environmental conditions, rather than spatial factors, such as latitude or elevation, best predicted the variance of community composition and diversity [44, 45]. Suggesting that the differences in microbial community here were mainly led by the variance in habitat and environmental

344 characteristic in the rivers, the longitudinal natural changes in rivers may account for some of the
345 environmental and biological variation observed [46].

346 *4.1. Habitat Restoration Impact on Physico-chemical Properties of Stream Water*

347 The consistent input of pollutants from both point and diffuse sources in the urban (pre-
348 restored) rivers caused high enrichment of TOC. Habitat restoration led to a reduction in TOC, and a
349 significant increase in DO in the surface water of the restored rivers. These results are consistent with
350 habitat restoration experiments in the Zenne River in Belgium [47]. Essentially, habitat restoration
351 improved conditions by reducing TOC and increasing DO, suggesting that organic pollutants
352 entering the degraded river were removed through habitat restoration. There was no difference in
353 DO concentration between restored and reference forest rivers, suggesting that habitat restoration
354 improved the physico-chemical environment of restored rivers.

355 *4.2. Impact of Habitat Restoration on the Bacterial Community*

356 The diversity and composition of bacterial communities change according to habitat
357 characteristics [48], hence, rehabilitation methods and the intensity of application should affect both
358 the composition and diversity of microbial communities. Here, no differences were detected among
359 river types for bacterial richness, and a significant decline in bacterial diversity was detected in
360 restored rivers compared to degraded rivers. This is consistent with studies in wastewater treatment
361 plant (WWTP) effluent in both urban and rural areas where a reduced diversity of biofilm bacteria
362 has been detected [49, 50]. The difference in bacterial diversity might reflect the physico-chemical
363 variables of surface water in the different river types. Dissolved inorganic nitrogen, dissolved organic
364 carbon and hydrological variability have been demonstrated to be the most important environmental
365 factors affecting biofilm responses [51]. In this study, the increase of DO concentration caused by
366 habitat restoration might lead to the development of aerobic microbial community and higher
367 efficiencies of chemical oxygen demand removal through oxidative decomposition [52]. The decline
368 in organic carbon quality could also influence the abundance of biofilm bacteria [51, 53], which might
369 have led to the decrease in heterotrophic anaerobic microorganism that rely on organic resources,
370 which lead to the decline of bacterial diversity in rivers after habitat restoration. Epilithic bacterial
371 populations can also be affected indirectly by inorganic nutrients via the influence of nutrients on
372 algal biomass [54, 55].

373 Distinct bacterial communities were detected in each of the river types, a dissimilar composition
374 was found between (i) forest rivers and degraded rivers, (ii) forest rivers and restored rivers, and (iii)
375 restored rivers and degraded rivers. These differences were strongly correlated with the changes in
376 habitat substrate diversity, and physico-chemical characteristics (DO, TOC and COD) of these river
377 types. The results from this study suggest that the differences in bacterial community compositions
378 were mainly caused by the variations in habitat and habitat-specific physico-chemical characteristics
379 [48, 56]. Rivers with diverse substrates may provide more dynamic surface and higher degree of
380 resource heterogeneity within the microhabitats for biofilms, shaping distinct bacterial communities
381 in forest and restored rivers from microbiome in degraded rivers. The variations in physico-chemical
382 attributes (e.g. TOC) in forest and restored rivers might led to the difference in bacterial community
383 composition between these two river types. Moreover, the bacteria clustered in the restored rivers
384 were distributed between the bacteria in the degraded and forest rivers, indicating that they were
385 moving in the correct direction, i.e. towards the reference forest rivers. There was, however, some
386 overlap between the restored and degraded rivers, indicating that there was still a legacy effect of the
387 previous degraded state. Overall, the degraded rivers possessed significantly greater bacterial
388 diversity than the restored rivers. Hence, restoration to "pristine" conditions will take longer than
389 seven years, and further studies are needed to determine exactly how long.

390 Compared with forest rivers, degraded rivers had a slightly greater abundance of Bacteroidetes,
391 a member of phylum specialized in degrading high molecular weight (HMW) compounds, and

possessed significantly higher relative abundance of *Flavobacterium*, *Arenimonas* and *Acinetobacter*, which are capable of metabolizing/mineralizing organic compounds [57-59], and a remarkably low abundance of *Duganella*, *Indobacter*, *Methylotenera*, *Rhodoferax* and *Clostridium_sensu_stricto_13*; these genera are major players in cycling of carbon compounds in the environment [60, 61], and organic matter utilization [62]. This suggests that the degraded rivers with a high TOC load and limited DO have a distinct impact on the microbial community, shaping the microbiome with a greater ability to degrade/mineralize high molecular weight (HMW) compounds in degraded rivers; this ability differentiates these degraded rivers from the forest ones.

The restored rivers, however, had a greater relative Proteobacteria abundance than degraded rivers; this phylum is often found in nutrient-poor conditions with a low TOC [47]. Moreover, *Duganella* genus which utilized organic compounds, but required oxygen to survive [63] was greater in restored rivers compared to the degraded ones. This may imply that along with the establishment of more diverse substrates and aerobic and sub-aerobic system in the restored rivers, habitat restoration shifted the dominant components of the bacterial community that mineralize and degrade organic matter to bacteria that utilize organic matter for growth. At the same time, there is also a shift from species that occur in predominantly anaerobic conditions to aerobic conditions. This is consistent with the RDA results, where the bacterial community in the degraded rivers was strongly correlated to organic pollutants TOC and COD, whereas, for restored rivers, the bacterial community only showed weak positive correlations with substrate diversity and DO in the surface water.

In terms of the relationship between restored rivers and forest rivers, no significant differences in bacterial diversity, bacterial richness, and relative abundance of the Proteobacteria and Bacteroidetes were found. However, restored rivers possessed a lower abundance of *Indobacter*, *Methylotenera*, *Rhodoferax* and *Clostridium_sensu_stricto_13* than forest rivers. Moreover, the *Flavobacterium*, *Pseudomonas* and *Acinetobacter* were found in greater abundance in degraded rivers were much greater in restored rivers compared to forest rivers. This suggests that restored rivers still possess species that degrade/mineralize the high concentrations of organic compounds that persist even after restoration. In summary, our results highlight effective dissolved oxygen enhancement, organic pollutants reduction trends, and alongside changes in the microbial community during river habitat restoration. However, restored rivers still have a long way to go to recover the natural status of pristine rivers, and continued monitoring is needed to measure the time scale required for the restored sites to attain the reference standards.

5. Conclusions

We examined the effect of habitat restoration on microbial community composition in biofilms using high-throughput 16S rRNA gene amplicon sequencing. The results showed that habitat restoration altered the bacterial community structure in a positive manner in the degraded rivers. Habitat restoration induced a lower bacterial diversity, but greater abundance of genera that degrade organic pollutants; these changes might be attributed to the status of dissolved oxygen and total organic carbon variables in the surface water. These results suggest that applying habitat restoration approaches to restore urban rivers by enhancing habitat heterogeneity, which can in turn alter the physico-chemical characteristics and stimulate the processing of organic pollutants through the variation of microbial community composition, which was moving in the right direction. Habitat restoration is, therefore, an efficient way for the switching of microbial community composition for sustainable freshwater restoration and management. It will take longer than seven years for degraded rivers to attain the similar ecosystem quality as the reference sites, and continued studies are needed to measure the time scale required for the recovery.

Author Contributions: Conceptualization, Q.L. and Y.Z.; methodology, Q.L. ; software, Q.L.; validation, Q.L. and Y.Z.; formal analysis, Q.L. and R.M.; investigation, Q.L.; resources, Y.Z.; data curation, Q.L.; writing—original draft preparation, Q.L.; writing—review and editing, Q.L., R.S., R.M. and Y.Z.; visualization, Q.L.; supervision, R.S., R.M. and Y.Z.; project administration, Y.Z.; funding acquisition, Y.Z and R.S.

Funding: This research was funded by Xi'an Jiaotong-Liverpool University (Grant number RDF-15-01-50), and Natural Science Foundation of Jiangsu Province (Grant number BK20171238). Raju Sekar would like to acknowledge the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (Grant No. 13KJB180022) and Natural Science Foundation of Jiangsu Province (Grant No. BK20141211) for funding support.

Acknowledgments: We would like to thank Prof. David Atkinson, Dr. Eduardo Medina-Roldan for their comments and suggestions for this field project, and thank stream group team members for their field experiment support, and thank Dr. Yi Zou, Williamson Gustave, Zhaofeng Yuan for their advice on statistical analysis.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Materials

Table S1. Detailed location data and habitat information for the nine study sites within the Anji City Region, PRC; Habitat information include canopy cover, habitat types, substrate composition and substrate Shannon index (H'). F = forest streams; R = restored streams; D = degraded streams.

Site code	River name	Location (Longitude & Latitude)	Canopy cover (%)	Habitat types present			Substrate composition (%)				Substrate Shannon Index(H')
				Island	Pool	Riffle	Boulders	Cobbles	Pebbles	Granules	
F-1	Longwang Mountain	30°25'3.93"N 119°24'30.52"E	70	✓	✓	✓	20.7	72	7	0.3	0.77
F-2	Yangjiao Mountain	30°26'59.18"N 119°27'55.03"E	90	✓	✓	✓	22.4	68.3	8.1	1.2	0.85
F-3	Zhebei Valley	30°25'24.05"N 119°30'33.60"E	85	✓	✓	✓	13.3	45.3	36.9	4.5	1.13
R-1	Shima Port	30°37'52.98"N 119°41'57.03"E	1	✓	✓	✓	0	13.3	38.7	48	0.99
R-2	Depu Port	30°36'22.34"N 119°41'39.80"E	2	✓	✓	✓	0	14.9	59.5	25.6	0.94
R-3	Wuxiangba	30°38'43.04"N 119°36'32.29"E	10	✓	✓	✓	0	68.5	29.7	1.8	0.69
D-1	Tongxin	30°38'13.96"N 119°41'28.86"E	20	-	✓	-	0	0	0	100	0
D-2	Wuzhuang	30°38'7.99"N 119°39'2.36"E	0.2	✓	✓	-	0	0	0	100	0
D-3	Chiyi	30°38'28.69"N 119°36'12.85"E	60	-	✓	-	0	0	0	100	0

References

1. Bernhardt, E.S.; Sudduth, E.B.; Palmer, M.A.; Allan, J.D.; Meyer, J.L.; Alexander, G.; Follastad-Shah, J.; Hassett, B.; Jenkinson, R.; Lave, R.; et al. Restoring rivers one reach at a time: Results from a survey of US river restoration practitioners. *Restor Ecol* **2007**, *15*, 482-493; DOI:10.1111/j.1526-100X.2007.00244.x.
2. Malmqvist, B.; Rundle, S. Threats to the running water ecosystems of the world. *Environ. Conserv.* **2002**, 29, 134–153; DOI: 10.1017/S0376892902000097.
3. Naiman, R.J.; Bunn, S.E.; Nilsson, C.; Petts, G.E.; Pinay, G.; Thompson, L.C. Legitimizing fluvial ecosystems as users of water: an overview. *Environ. Manage.* **2002**, 30, 455–467; DOI: 10.1007/s00267-002-2734-3.
4. Laasonen, P.; Muotka, T.; Kivijarvi, I. Recovery of macroinvertebrate communities from stream habitat restoration. *Aquat Conserv-Mar Freshw Ecosyst* **1998**, *8*, 101-113; DOI:10.1002/(SICI)1099-0755(199801/02)8:1<101::AID-AQC251>3.3.CO;2-W.
5. Lepori, F.; Palm, D.; Brannas, E.; Malmqvist, B. Does restoration of structural heterogeneity in streams enhance fish and macroinvertebrate diversity? *Ecol Appl* **2005**, *15*, 2060-2071; DOI:10.1890/04-1372.
6. Miller, S.W.; Budy, P.; Schmidt, J.C. Quantifying macroinvertebrate responses to in-stream habitat restoration: Applications of meta-analysis to river restoration. *Restor Ecol* **2010**, *18*, 8-19; DOI:10.1111/j.1526-100X.2009.00605.x.
7. Palmer, M.A.; Filoso, S.; Fanelli, R.M. From ecosystems to ecosystem services: Stream restoration as ecological engineering. *Ecol Eng* **2014**, *65*, 62-70; DOI:10.1016/j.ecoleng.2013.07.059.
8. Kail, J.; Brabec, K.; Poppe, M.; Januschke, K. The effect of river restoration on fish, macro-invertebrates and aquatic macrophytes: A meta-analysis. *Ecol Indic* **2015**, *58*, 311-321; DOI:10.1016/j.ecolind.2015.06.011.
9. Douglas, M.; Lake, P.S. Species richness of stream stones - An investigation of the mechanisms generating the species-area relationship. *Oikos* **1994**, *69*, 387-396; DOI:10.2307/3545851.
10. Palmer, M.A.; Allan, J.D.; Butman, C.A. Dispersal as a regional process affecting the local dynamics of marine and stream benthic invertebrates. *Trends Ecol Evol* **1996**, *11*, 322-326; DOI:10.1016/0169-5347(96)10038-0.
11. Lake, P.S. Disturbance, patchiness, and diversity in streams. *J N Am Benthol Soc* **2000**, *19*, 573-592; DOI:10.2307/1468118.
12. Louhi, P.; Mykra, H.; Paavola, R.; Huusko, A.; Vehanen, T.; Maki-Petays, A.; Muotka, T. Twenty years of stream restoration in Finland: Little response by benthic macroinvertebrate communities. *Ecol Appl* **2011**, *21*, 1950-1961; DOI:10.1890/10-0591.1.
13. Simaika, J.P.; Stoll, S.; Lorenz, A.W.; Thomas, G.; Sundermann, A.; Haase, P. Bundles of stream restoration measures and their effects on fish communities. *Limnologica* **2015**, *55*, 1-8; DOI:10.1016/j.limno.2015.10.001.
14. Flores, L.; Giorgi, A.; Gonzalez, J.M.; Larranaga, A.; Diez, J.R.; Elosegi, A. Effects of wood addition on stream benthic invertebrates differed among seasons at both habitat and reach scales. *Ecol Eng* **2017**, *106*, 116-123; DOI:10.1016/j.ecoleng.2017.05.036.
15. Nuttle, T.; Logan, M.N.; Parise, D.J.; Foltz, D.A.; Silvis, J.M.; Haibach, M.R. Restoration of macro-invertebrates, fish, and habitats in streams following mining subsidence: Replicated analysis across 18 mitigation sites. *Restor Ecol* **2017**, *25*, 820-831; DOI:10.1111/rec.12502.
16. Lear, G.; Ancion, P.Y.; Harding, J.; Lewis, G.D. Use of bacterial communities to assess the ecological health of a recently restored stream. *N Z J Mar Freshw Res* **2012**, *46*, 291-301; DOI:10.1080/00288330.2011.638647.
17. Gantzer, C.J.; Rittmann, B.E.; Herricks, E.E. Effect of long-term water velocity changes on streambed biofilm activity. *Water Res* **1991**, *25*, 15-20; DOI:10.1016/0043-1354(91)90093-6.
18. Lawrence, J.R.; Chenier, M.R.; Roy, R.; Beaumier, D.; Fortin, N.; Swerhone, G.D.W.; Neu, T.R.; Greer, C.W. Microscale and molecular assessment of impacts of nickel, nutrients, and oxygen level on structure and function of river biofilm communities. *Appl Environ Microbiol* **2004**, *70*, 4326-4339; DOI:10.1128/AEM.70.7.4326-4339.2004.
19. Lear, G.; Anderson, M.J.; Smith, J.P.; Boxen, K.; Lewis, G.D. Spatial and temporal heterogeneity of the bacterial communities in stream epilithic biofilms. *FEMS Microbiol Ecol* **2008**, *65*, 463-473; DOI:10.1111/j.1574-6941.2008.00548.x.
20. Battin, T.J.; Besemer, K.; Bengtsson, M.M.; Romani, A.M.; Packmann, A.I. The ecology and biogeochemistry of stream biofilms. *Nat Rev Microbiol* **2016**, *14*(4), 251-263; DOI:10.1038/nrmicro.2016.15.
21. Fischer, H.; Sukhodolov, A.; Wilczek, S.; Engelhardt, C. Effects of flow dynamics and sediment movement on microbial activity in a lowland river. *River Res Appl* **2003**, *19*, 473-482; DOI:10.1002/rra.731.

22. Sheldon, F.; Walker, K.F. Changes in biofilms induced by flow regulation could explain extinctions of aquatic snails in the lower River Murray, Australia. *Hydrobiologia* **1997**, *347*, 97-108; DOI:10.1023/a:1003019302094.
23. Cotner, J.B.; Biddanda, B.A. Small players, large role: Microbial influence on biogeochemical processes in pelagic aquatic ecosystems. *Ecosystems* **2002**, *5*, 105-121; DOI:10.1007/s10021-001-0059-3.
24. Battin, T.J.; Kaplan, L.A.; Newbold, J.D.; Cheng, X.H.; Hansen, C. Effects of current velocity on the nascent architecture of stream microbial biofilms. *Appl Environ Microbiol* **2003**, *69*, 5443-5452; DOI:10.1128/AEM.69.9.5443–5452.2003.
25. Zeglin, L.H. Stream microbial diversity in response to environmental changes: Review and synthesis of existing research. *Front Microbiol* **2015**, *6*; DOI:10.3389/fmicb.2015.00454.
26. Valett, H.M.; Thomas, S.A.; Mulholland, P.J.; Webster, J.R.; Dahm, C.N.; Fellows, C.S.; Crenshaw, C.L.; Peterson, C.G. Endogenous and exogenous control of ecosystem function: N cycling in headwater streams. *Ecology* **2008**, *89*, 3515-3527; DOI:10.1890/07-1003.1.
27. Mulholland, P.J.; Helton, A.M.; Poole, G.C.; Hall, R.O.; Hamilton, S.K.; Peterson, B.J.; Tank, J.L.; Ashkenas, L.R.; Cooper, L.W.; Dahm, C.N. Stream denitrification across biomes and its response to anthropogenic nitrate loading. *Nature* **2008**, *452*, 202-U246; DOI:10.1038/nature06686.
28. Violin, C.R.; Cada, P.; Sudduth, E.B.; Hassett, B.A.; Bernhardt, P.E.S. Effects of urbanization and urban stream restoration on the physical and biological structure of stream ecosystems. *Ecol. Appl.* **2011**, *21*(6), 1932-1949.
29. Kondolf, G.M. Application of the pebble count: Notes on purpose, method, and variants. *J Am Water Resour Assoc* **1997**, *33*, 79-87; DOI:10.1111/j.1752-1688.1997.tb04084.x.
30. Shannon, C.E. The mathematical theory of communication (Reprinted). *M D Comput* **1997**, *14*, 306-317.
31. Elizabeth, J.; Arar, G.B.C. Method 445.0 In vitro determination of chlorophyll a and pheophytin a in marine and freshwater algae by fluorescence. **1997**.
32. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Microbiol Biotechnol* **2007**, *73*(16), 5261-5267; DOI:10.1128/AEM.00062-07.
33. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*(16), 2194-2200; DOI:10.1093/bioinformatics/btr381.
34. Torres-Mellado, G.A.; Escobar, I.; Palfner, G.; Casanova-Katny, M.A. Mycotrophy in Gilliesieae, a threatened and poorly known tribe of Alliaceae from central Chile. *Rev Chil Hist Nat* **2012**, *85*, 179-186; DOI:10.4067/S0716-078X2012000200004.
35. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. **2017**. URL https://www.R-project.org/.
36. Wang, Y.J.; Zhang, H.; Zhu, L.; Xu, Y.L.; Liu, N.; Sun, X.M.; Hu, L.P.; Huang, H.; Wei, K.; Zhu, R.L. Dynamic distribution of gut microbiota in goats at different ages and health states. *Front Microbio* **2018**, *l 9*; DOI:10.3389/fmicb.2018.02509.
37. Oksanen, J.F.; Blanchet, G.; Friendly, M.; Kindt, R.; Legendre, P.; Mcglinn, D.; Minchin, P.; Hara, R.; Simpson, G.; Solymos, P.; et al. Package 'vegan', community ecology package. **2018**.
38. White, J.R.; Nagarajan, N.; Pop, M. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PLoS Comput Biol* **2009**, *5*, e1000352; DOI:10.1371/journal.pcbi.1000352.
39. Ter Braak, C.J.F. CANOCO - An extension of decorana to analyze species-environment relationships. *Vegetatio* **1988**, *75*, 159-160.
40. Fernandez-Gomez, B.; Richter, M.; Schueler, M.; Pinhassi, J.; Acinas, S.G.; Gonzalez, J.M.; Pedros-Alio, C. Ecology of marine Bacteroidetes: A comparative genomics approach. *ISME J* **2013**, *7*, 1026-1037; DOI:10.1038/ismej.2012.169.
41. Geist, J.; Hawkins, S.J. Habitat recovery and restoration in aquatic ecosystems: Current progress and future challenges. *Aquat Conserv-Mar Freshw Ecosyst* **2016**, *26*, 942-962; DOI:10.1002/aqc.2702.
42. Shrestha, S.; Farrelly, J.; Eggleton, M.; Chen, Y.S. Effects of conservation wetlands on stream habitat, water quality and fish communities in agricultural watersheds of the lower Mississippi River Basin. *Ecol Eng* **2017**, *107*, 99-109; DOI:10.1016/j.ecoleng.2017.06.054.
43. Fisher, S.G. Stream ecology - Structure and function of running waters - Allan, Jd. *Science* **1995**, *270*, 1858-1858.

44. Lear, G. et al. The biogeography of stream bacteria. *Glob. Ecol. Biogeogr.* **2013**, 22, 544–554; DOI: /10.1111/geb.12046.
45. Fierer, N.; Morse, J.L.; Berthrong, S.T.; Bernhardt, E.S.; Jackson, R.B. Environmental controls on the landscape-scale biogeography of stream bacterial communities. *Ecology* **2007**, 88, 2162–2173; DOI: 10.1890/06-1746.1.
46. Vannote, R.L.; Minshall, G.W.; Cummins, K.W.; Sedell, J.R.; Cushing, C.E. The river continuum concept. *Can. J. Fish. Aquat. Sci.* **1980,** 37(1), 130-137; DOI: 10.1139/f80-017.
47. Atashgahi, S.; Aydin, R.; Dimitrov, M.R.; Sipkema, D.; Hamonts, K.; Lahti, L.; Maphosa, F.; Kruse, T.; Saccenti, E.; Springael, D.; et al. Impact of a wastewater treatment plant on microbial community composition and function in a hyporheic zone of a eutrophic river. *Sci Rep* **2015**, *5*, 17284; DOI:10.1038/srep17284.
48. Levi, P.S.; Starnawski, P.; Poulsen, B.; Baattrup-Pedersen, A.; Schramm, A.; Riis, T. Microbial community diversity and composition varies with habitat characteristics and biofilm function in macrophyte-rich streams. *Oikos* **2016**; DOI:10.1111/oik.03400.
49. Drury, B.; Rosi-Marshall, E.; Kelly, J.J. Wastewater treatment effluent reduces the abundance and diversity of benthic bacterial communities in urban and suburban rivers. *Appl Environ Microbiol* **2013**, *79*, 1897–1905; DOI:10.1128/Aem.03527-12.
50. Lu, X.M.; Lu, P.Z. Characterization of bacterial communities in sediments receiving various wastewater effluents with high-throughput sequencing analysis. *Microb Ecol* **2014**, *67*(3), 612; DOI:10.1007/s00248-014-0370-0.
51. Ponsatí, L.; Corcoll, N.; Petrović, M.; Picó, Y.; Ginebreda, A.; Tornés, E.; Guasch, H.; Barcelo, D.; Sabater, S. Multiple-stressor effects on river biofilms under different hydrological conditions. *Freshw Biol* **2016**, *61*, 2102-2115; DOI:10.1111/fwb.12764.
52. Gu, D.G.; Xu, H.; He, Y.; Zhao, F.; Huang, M.S. Remediation of urban river water by pontederia cordata combined with artificial aeration: Organic matter and nutrients removal and root-adhered bacterial communities. *Int J Phytoremediat* **2015**, *17*, 1105-1114; DOI:10.1080/15226514.2015.1045121.
53. Olapade, O.A.; Leff, L.G. Seasonal response of stream biofilm communities to dissolved organic matter and nutrient enrichments. *Appl Environ Microbiol* **2005**, *71*, 2278-2287; DOI:10.1128/AEM.71.5.2278-2287.2005.
54. Rier, S.T.; Stevenson, R.J. Effects of light, dissolved organic carbon, and inorganic nutrients on the relationship between algae and heterotrophic bacteria in stream periphyton. *Hydrobiologia* **2002**, *489*, 179-184; DOI:10.1023/A:1023284821485.
55. Tank, J.L.; Webster, J.R. Interaction of substrate and nutrient availability on wood biofilm processes in streams. *Ecology* **1998**, *79*, 2168-2179; DOI:10.1890/0012-9658(1998)079[2168:IOSANA]2.0.CO;2.
56. Hempel, M.; Grossart, H.P.; Gross, E.M. Community composition of bacterial biofilms on two submerged macrophytes and an artificial substrate in a pre-alpine Lake. *Aquat. Microb. Ecol.* **2010**, 58, 79–94; DOI: 10.3354/ame01353.
57. Verma, D.K.; Rathore, G. New host record of five Flavobacterium species associated with tropical fresh water farmed fishes from North India. *BJM* **2015**, *46*(4), 969-76; DOI:10.1590/S1517-838246420131081.
58. Chen, F.; Wang, H.; Cao, Y.J.; Li, X.Y.; Wang, G.J. High quality draft genomic sequence of Arenimonas donghaensis DSM 18148(T). *Stand Genomic Sci* **2015**, *10*, 59; DOI: 10.1186/s40793-015-0055-4.
59. Garcia-Garcera, M.; Touchon, M.; Brisse, S.; Rocha, E. Metagenomic assessment of the interplay between the environment and the genetic diversification of Acinetobacter. *Environ Microbiol* **2017**, *19*(12), 5010-5024; DOI:10.1111/1462-2920.13949.
60. Vorobev, A.; Beck, D.A.; Kalyuzhnaya, M.G.; Lidstrom, M.E.; Chistoserdova, L. Comparative transcriptomics in three Methylophilaceae species uncover different strategies for environmental adaptation. *PeerJ* **2013**, *1*, e115; DOI:10.7717/peerj.115.
61. Risso, C.; Sun, J.; Zhuang, K.; Mahadevan, R.; DeBoy, R.; Ismail, W.; Shrivastava, S.; Huot, H.; Kothari, S.; Daugherty, S. Genome-scale comparison and constraint-based metabolic reconstruction of the facultative anaerobic Fe(III)-reducer Rhodoferax ferrireducens. *BMC genomics* **2009**, *10*, 447; DOI:10.1186/1471-2164-10-447.
62. Zhao, D.; Cao, X.; Huang, R.; Zeng, J.; Wu, Q.L. Variation of bacterial communities in water and sediments during the decomposition of Microcystis biomass. *PLoS One* **2017**, *12*; DOI:10.1371/journal.pone.0176397.
63. Kämpfer, P.; Wellner, S.; Lohse, K.; Martin, K.; Lodders, N. Duganella phyllosphaerae sp. nov. isolated from the leaf surface of trifolium repens and proposal to reclassify duganella violaceinigra into a novel genus as

615 pseudoduganella violceinigra gen. nov. comb. nov. Syst. *Appl Microbiol* **2012**, *35*(1), 19-23;
616 DOI:10.1016/j.syapm.2011.10.003.
617

618